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Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions

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ABSTRACT The thermophilic, peptidolytic, anaerobic bacterium *Clostridium fervidus* is unable to generate a pH gradient in the range of 5.5–8.0, which limits growth of the organism to a narrow pH range (6.3–7.7). A significant membrane potential ($\Delta\psi \approx -60$ mV) and chemical gradient of Na^+ ($-\Delta\mu_{\text{Na}} \approx -60$ mV) are formed in the presence of metabolizable substrates. Energy-dependent Na^+ efflux is inhibited by the Na^+/H^+ ionophore monensin but is stimulated by uncouplers, suggesting that the Na^+ gradient is formed by a primary pumping mechanism rather than by secondary Na^+/H^+ antiport. This primary sodium pump was found to be an ATPase that has been characterized in inside-out membrane vesicles and in proteoliposomes in which solubilized ATPase was reconstituted. The enzyme is stimulated by Na^+ , resistant to vanadate, and sensitive to nitrate, which is indicative of an F/V-type Na^+ -ATPase. In the proteoliposomes Na^+ accumulation depends on the presence of ATP, is inhibited by the ATPase inhibitor nitrate, and is completely prevented by the ionophore monensin but is stimulated by protonophores and valinomycin. These and previous observations, which indicated that secondary amino acid transport uses solely Na^+ as coupling ion, demonstrate that energy transduction at the membrane in *C. fervidus* is exclusively dependent on a Na^+ cycle.

Clostridium fervidus is a Gram-positive, spore-forming, thermophilic anaerobe that has been isolated from a hot spring in New Zealand. The organism ferments peptides and amino acids, yielding acetate, NH_3 , CO_2 , and H_2 as major end products (ref. 1; G.S., unpublished). Amino acid transport in this organism is coupled exclusively to sodium ions, indicating that the electrochemical sodium ion gradient ($\Delta\mu_{\text{Na}^+}$) plays a prominent role in the uptake of substrates essential for growth (2, 3). In accordance, growth of *C. fervidus* is completely inhibited by the Na^+/H^+ ionophore monensin and is only partially inhibited by protonophores (G.S., unpublished).

In a strictly fermentative bacterium such as *C. fervidus* the generation of a sodium gradient is most easily envisaged by (i) a H^+ -pumping ATPase in combination with a Na^+/H^+ antiporter, (ii) a membrane-bound Na^+ -pumping decarboxylase (4, 5), or (iii) a Na^+ -pumping ATPase (6). To establish how the Na^+ gradient of *C. fervidus* is generated, the ion gradients across the cytoplasmic membrane have been estimated in intact cells and the Na^+ -pumping mechanism has been characterized in inside-out membrane vesicles and proteoliposomes. An F/V-type Na^+ -ATPase is detected that functions *in vivo* as a Na^+ -extruding ATP hydrolase. To our knowledge, a primary Na^+ pump for a thermophilic eubacterium has not been reported previously. *C. fervidus* appears to be unique in the sense that energy transduction across the membrane is exclusively dependent on Na^+ and that no H^+ cycle is present.

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METHODS

Growth of the Organism and Preparation of Inside-Out Membrane Vesicles. *C. fervidus* ATCC 43204 was grown anaerobically at pH 7.0 and 68°C in tryptone/yeast extract/glucose (TYEG) medium as described (3). Cells were harvested in the mid-exponential phase of growth ($A_{660} = 0.3$ – 0.35). Inside-out membrane vesicles were prepared as described (7) with the following modifications: 3-(*N*-Morpholino)propanesulfonic acid (Mops) was used as a buffer, EDTA was omitted, and MgSO_4 (5 mM) and DNase and RNase (20 $\mu\text{g}/\text{ml}$, each) were added prior to passage through a French press cell.

Determination of Transmembrane Ion Gradients. *C. fervidus* cells were harvested in the exponential phase of growth, washed once with basal medium supplemented with 5 mM NaCl and 3 mM dithiothreitol (MMND), and resuspended to an A_{660} of 1.0 into 4 ml of the same medium. All operations were performed at room temperature and with the aid of an anaerobic cabinet. Cells were incubated for 10 min at 68°C with 10 mM glucose or arginine and the appropriate radioactive probes. The membrane potential ($\Delta\psi$), the pH gradient (ΔpH), and the $\Delta\mu_{\text{Na}^+}$ were estimated by the filtration and silicon oil centrifugation method (2, 8). [^3H]Tetraphenylphosphonium ion (TPP^+), [^{14}C]benzoic acid, [^{14}C]methylamine, and α -amino [^{14}C]isobutyrate (AIB) were used at final concentrations of 5 μM , 5 μM , 4 μM , and 5 μM , respectively. Samples to which 10 μM valinomycin and 1 μM nigericin were added to fully dissipate the $\Delta\psi$ and ΔpH served as blanks for the estimation of the gradients. In parallel experiments, the internal volume was determined from the difference in the distribution of $^3\text{H}_2\text{O}$ and [^{14}C]sorbitol. The internal volume of *C. fervidus* was 1.85 $\mu\text{l}/\text{mg}$ of protein.

$^{22}\text{Na}^+$ Efflux. Cells of *C. fervidus* were handled as described above and resuspended to a final A_{660} of about 13 into MMND and equilibrated with 5 mM $^{22}\text{Na}^+$ overnight at room temperature. $^{22}\text{Na}^+$ efflux was assayed by the filtration method (2).

ATPase Activity. ATP hydrolysis was determined as described (9). Measurements were performed at 45°C in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes; pH 6.0) containing 1 mM MgCl_2 , 50 mM NaCl, plus 50 mM KCl for the inside-out membrane vesicles, and in 50 mM Mops (pH 7.0)

Abbreviations: $\Delta\mu_{\text{Na}^+}$, electrochemical gradient of sodium ions (in mV); $\Delta\mu_{\text{Na}}$, concentration gradient of sodium ions; $\Delta\mu_{\text{H}^+}$, proton motive force (in mV); ΔpH , pH gradient; *Z*, 2,3RT/*F*, conversion factor to express pH or Na^+ concentration gradients (in mV); AIB, α -aminoisobutyrate; $\Delta\mu_{\text{AIB}}$, concentration gradient of AIB (in mV); $\Delta\psi$, membrane potential (in mV); TPP^+ , tetraphenylphosphonium ion; S13, 5-chloro-3-*tert*-butyl-2'-chloro-4'- NO_2 -salicylanilide; SF-6847, 3,5-di(*tert*-butyl-4-hydroxybenzylidene) malonitrile.

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containing 100 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (buffer A) plus 50 mM NaCl for the proteoliposomes.

Reconstitution of the ATPase. Inside-out membrane vesicles (4 mg of protein) were solubilized for 10 min at 45°C in 2.5 ml of buffer A containing 1% (wt/wt) Triton X-100 plus 20% (wt/wt) glycerol. The Triton X-100 extract was centrifuged for 60 min at 4°C at 48,000 × *g*. The supernatant, preformed liposomes, and Triton X-100 were mixed as described (10). The mixture was equilibrated for 2 h at 4°C. Two times 2 g (for 3 and 16 h) and one times 4 g (for 3 h) of Bio-Beads were added per 200 mg of Triton X-100. The mixture was centrifuged for 2 h at 4°C at 200,000 × *g*. The liposomes were resuspended in buffer A to about 70 mg of phospholipid per ml and stored at 4°C.

²²Na⁺ Uptake in Proteoliposomes. Proteoliposomes were diluted into 1 ml of buffer A (0.1 mg of protein per ml, final concentration). ²²Na⁺ was added (0.5 mM, final concentration) and allowed to equilibrate. Tris-ATP (3 mM, final concentration) was used as substrate. Samples of 100 μl were taken and assayed for ²²Na⁺ uptake by the filtration method (2).

Other Methods. Protein was determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard. Liposomes were prepared as described (3) in buffer A.

Materials. The following radioactively labeled materials were purchased from Amersham: [³H]TPP⁺ (92.5 GBq/mol), [¹⁴C]benzoic acid (0.81 TBq/mol), [¹⁴C]methylamine (2.07 TBq/mol), ³H₂O (37 GBq/l), [¹⁴C]sorbitol (12.3 TBq/mol), [¹⁴C]AIB (2.18 TBq/mol), and ²²Na⁺ (27.2 TBq/mol).

RESULTS

Effect of pH on Growth and Energization. The effect of the initial medium pH on growth of *C. fervidus* is shown in Fig. 1. Growth rates and maximal optical densities sharply decreased below pH 6.3 and above pH 7.7. No growth was observed at or below pH 6.0 and at or above pH 8.0. This observation and the observations that amino acid transport is exclusively coupled to Na⁺ led us to estimate the ΔpH, the Δψ, and ΔpNa⁺ in intact cells in the presence of glucose or arginine and at various pH values (Table 1). The ΔpAIB has been used to estimate the ΔpNa⁺, by assuming that the

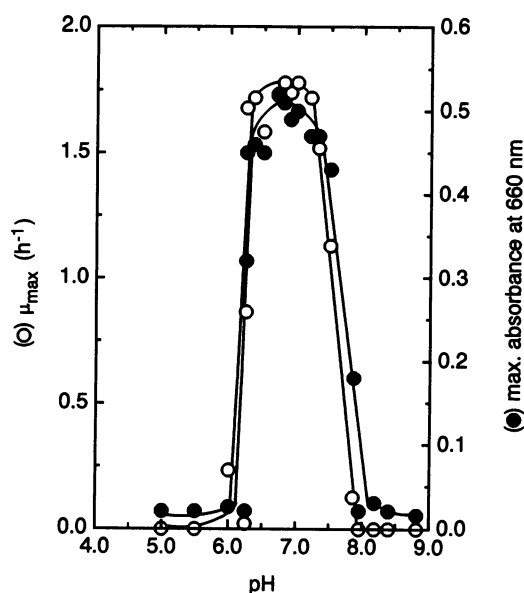


FIG. 1. pH dependence of growth of *C. fervidus*. Cells were grown at 68°C in TYEG medium adjusted to various pH values with HCl or KOH.

Table 1. Transmembrane ion gradients in *C. fervidus*

	Ion gradient, mV		
	Glucose		Arginine, pH 7.0
	pH 5.5	pH 7.0	pH 8.0
−ZΔpH*	−8	−11	+18
−ZΔpH†	0	+2	−13
Δψ	−60	−63	−73
ΔpAIB	−109	−124	−118
−ZΔpNa	−49	−61	−45

Z, 2.3RT/F, conversion factor to express pH or H⁺ concentration gradients (in mV); ΔpAIB, concentration gradient of AIB (in mV); ΔpNa, concentration gradient of sodium ions. Steady-state accumulations were determined at 68°C. The distributions of [³H]TPP⁺, [¹⁴C]AIB, [¹⁴C]benzoic acid, and [¹⁴C]methylamine were used to estimate the Δψ, ΔpAIB, −ZΔpH (inside alkaline), and −ZΔpH (inside acid), respectively. The −ZΔpNa was calculated by subtracting the Δψ from the ΔpAIB.

*Results determined from the distribution of benzoic acid.

†Results determined from the distribution of methylamine.

steady-state transmembrane gradient of AIB (ΔpAIB) is in thermodynamic equilibrium with the electrochemical sodium gradient. The ZΔpNa was obtained by subtracting the Δψ from the ΔpAIB. A significant ΔpH, either internally alkaline or acidic relative to the outside, could not be detected in the pH range 5.5–8.0, irrespective of whether glucose or arginine served as energy source. The ΔpH assay used was found to be sufficiently sensitive to detect artificially imposed pH gradients of 0.3 unit (−ZΔpH is −20 mV) (G.S., unpublished). However, under these conditions a large Δψ, ΔpNa, and ΔpNa⁺ were formed, and these gradients were relatively constant in the pH range 5.5–8.0 (Table 1). No significant differences were found between the gradients estimated with the filtration or silicone oil centrifugation method. The observation that *C. fervidus* is unable to generate a ΔpH and to regulate the cytoplasmic pH is consistent with the observed narrow pH range of growth.

Effect of Ionophores on Transmembrane Ion Gradients. The electrochemical gradient of Na⁺ in *C. fervidus* can be generated by two distinct mechanisms that are shown in Fig. 2. In Fig. 2A, a proton motive force (in mV) (ΔpH₊) is formed by the activity of a H⁺-ATPase and this ΔpH₊ is converted into a ΔpNa⁺ by a H⁺/Na⁺ antiporter system. Fig. 2B shows a Na⁺-ATPase as the mechanism of Na⁺ excretion. To discriminate between these two mechanisms of ΔpNa⁺ generation, the effect of ionophores on Na⁺ efflux in intact cells was studied. Arginine was used as an energy source since it is taken up in exchange for ornithine without net movement of ²²Na⁺, and intracellular ATP is formed rapidly by substrate level phosphorylation (3). Resting cells equilibrated with ²²Na⁺ displayed little or no efflux of Na⁺. Upon addition of arginine ²²Na⁺ was extruded (Fig. 3A), and after correction for binding, a 7- to 10-fold decrease of the internal Na⁺ concentration could be calculated, yielding a −ZΔpNa of −50 to −60 mV. These values are in agreement with those presented in Table 1. ²²Na⁺ efflux was stimulated by the protonophore S13 but was inhibited by monensin (Fig. 3A). After energization monensin caused an immediate influx of Na⁺, whereas S13 had less effect than the ethanol control (Fig. 3B). These results are at variance with the model of Fig. 2A: The protonophore would dissipate the proton motive force, thereby decreasing the Na⁺/H⁺ antiporter activity, which indirectly results in the dissipation of a Na⁺ gradient. The electroneutral Na⁺/H⁺ exchanger monensin would serve as a substitute for a Na⁺/H⁺ antiporter and convert a pH gradient into a sodium gradient or vice versa, depending on the magnitude and direction of these gradients. According to the model in Fig. 2B, dissipation of the Δψ by protonophores would lead to an increase of the chemical gradient of sodium due to additional pumping of Na⁺ by the

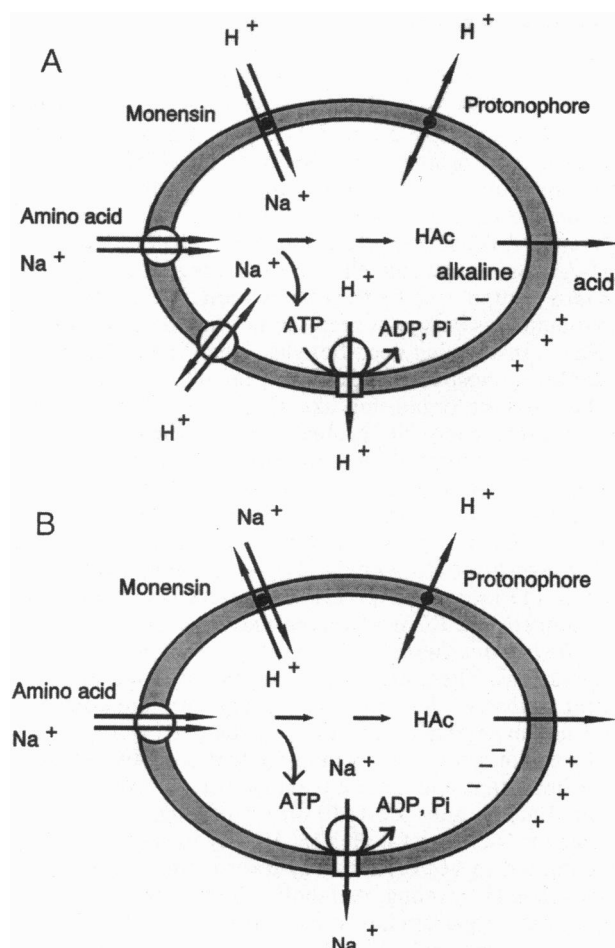


FIG. 2. Tentative schemes for H⁺ and Na⁺ fluxes in *C. fervidus* and the actions of ionophores: Generation of a Na⁺ gradient by the action of a H⁺-ATPase together with a Na⁺/H⁺ antiporter (A) or a Na⁺-ATPase (B). The effects of ionophores and protonophores are explained in the text.

ATPase. Monensin would convert the Na⁺ gradient into a pH gradient. These are indeed the effects that are observed in *C. fervidus*. These results are also consistent with previous ones

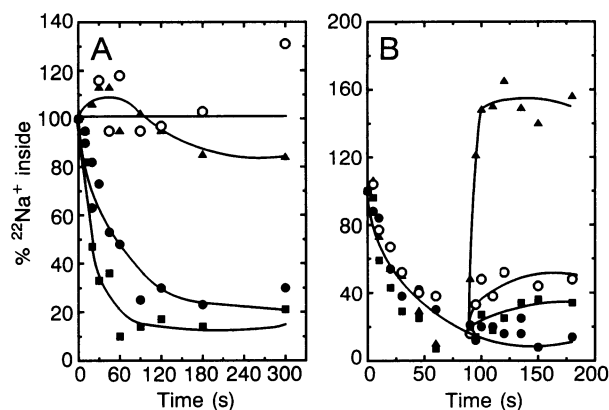


FIG. 3. ²²Na⁺ efflux from cells of *C. fervidus*. Cells were harvested, washed, and equilibrated with ²²Na⁺ (5 mM, 74 MBq/liter). (A) Cells were incubated for 5 min in the absence (○, ●) or presence (■) of 20 μM 5-chloro-3-tert-butyl-2'-chloro-4'-NO₂-salicylanilide (S13) or 2 μM monensin (▲). The experiment was started by the addition of 5 mM arginine (■, ●, ▲) or no addition (○). (B) ²²Na⁺ efflux was started by the addition of 5 mM arginine. At *t* = 90 s, 2% ethanol (○), 20 μM S13 (■), or 2 μM monensin was added (▲) or no addition was made (●).

in which in membrane vesicles no Na⁺/H⁺ but only Na⁺/Na⁺ exchange activity was observed (3). Since ATP production from the metabolism of arginine is able to elicit the Na⁺ extrusion, the primary Na⁺-pump mechanism most likely is a Na⁺-ATPase.

Effect of Monovalent Cations on ATP Hydrolysis. Inhibitor and immunological studies indicated the presence of an F/V-type ATPase in membranes of *C. fervidus* (G.S., unpublished). This ATPase was studied in inside-out membrane vesicles and in proteoliposomes in which solubilized ATPase was reconstituted. The effect of increasing NaCl concentrations (at constant ionic strength) on ATP hydrolysis is shown in Fig. 4. The stimulation by Na⁺ ions shows Michaelis-Menten kinetics from which an apparent *K_m* (Na⁺) of 3.2 mM can be estimated. The "zero" concentration in Fig. 4 reflects ≈25 μM (contaminating) Na⁺ (data not shown). This activation of ATPase activity by Na⁺ is indicative of a Na⁺-translocating ATPase.

Functional Reconstitution of ATPase Activity into Liposomes. The reconstituted ATPase activity exhibits the same Na⁺ dependency as the ATPase activity in inside-out membrane vesicles (Fig. 4). Again, an apparent *K_m* of about 3 mM can be estimated for the activation by Na⁺. The reconstituted ATPase exhibits properties identical to the ATPase activity in inside-out membrane vesicles (Table 2). Upon lowering the pH from 7.0 to 6.0 the activity is 2-fold stimulated. NO₃⁻ inhibits the ATPase activity, SO₃²⁻ activates, whereas orthovanadate has no effect on the enzyme (Table 2). ATP hydrolysis in the proteoliposomes was stimulated by valinomycin, indicating that a Δ*ψ* is formed. Triton X-100 had a far more stimulating effect on ATP hydrolysis in the proteoliposomes than in the inside-out membrane vesicles, indicating that in proteoliposomes the ATPase is for the major part oriented with the binding side for ATP on the inside.

ATP-Driven ²²Na⁺ Uptake in Proteoliposomes. ²²Na⁺ was accumulated into the proteoliposomes upon the addition of ATP (Fig. 5). This accumulation was dependent on ATPase activity, since in the absence of ATP or in the presence of the ATPase inhibitor KNO₃ only equilibration of ²²Na⁺ was observed (Fig. 5A). The effects of ionophores and protono-

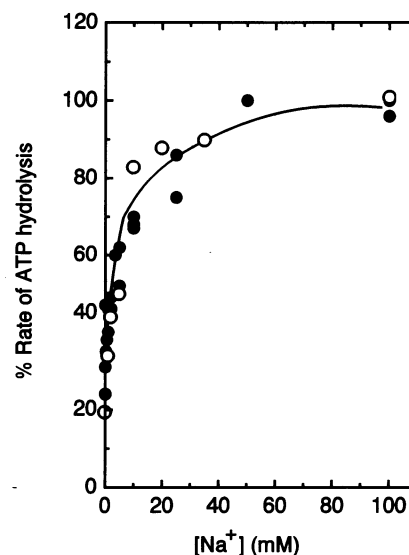


FIG. 4. Effect of increasing Na⁺ concentration on the ATP-hydrolyzing activity in inside-out membrane vesicles (●) and proteoliposomes (○). P_i release upon the addition of 2 mM Tris-ATP was measured in the absence of Triton X-100. The ionic strength was kept constant with KCl. Inside-out membrane vesicles were at pH 6.0 and proteoliposomes were at pH 7.0. One hundred percent activities represent 175 nmol and 59 nmol of P_i per min per mg of protein for inside-out membrane vesicles and proteoliposomes, respectively.

Table 2. Na^+ -ATPase activity in inside-out membrane vesicles and proteoliposomes

Addition	pH	Rate of ATP hydrolysis, %			
		Inside-out vesicles		Proteoliposomes	
		Without TX-100	With TX-100	Without TX-100	With TX-100
None (control)	7.0	100	100	100	100
None (control)	6.0	190	200	ND	182
KNO_3 (50 mM)	7.0	ND	ND	10	ND
KNO_3 (50 mM)	6.0	8	40	ND	ND
Na_2SO_3 (25 mM)	7.0	ND	ND	375	ND
Na_2SO_3 (25 mM)	6.0	400	ND	ND	ND
No NaCl	7.0	24	ND	18	ND
No NaCl	6.0	50	92	ND	ND
Valinomycin (4 μM)	7.0	ND	ND	260	ND
Vanadate (200 μM)	6.0	104	98	ND	ND

One hundred percent values for ATPase activity in inside-out membrane vesicles in the absence and presence of 0.05% Triton X-100 (TX-100) and in proteoliposomes in the absence or presence of 0.1% Triton X-100 correspond to 59, 88, 38, and 657 nmol of P_i per min per mg of protein, respectively. ATPase measurements were performed at 45°C at a Na^+ concentration of 50 mM. ND, not determined.

phores on $^{22}\text{Na}^+$ accumulation are shown in Fig. 5B. Monensin, which exchanges Na^+ for H^+ , prevented accumulation, whereas the protonophore SF-6847, which dissipates the ΔpH , and the ionophore valinomycin, which dissipates the $\Delta\psi$, stimulated $^{22}\text{Na}^+$ accumulation. This indicates that Na^+ uptake is an electrogenic and primary transport event. Control experiments demonstrated that SF-6847 and valinomycin completely dissipated the ΔpH and $\Delta\psi$, respectively (data not shown). ATPase activity reconstituted into liposomes was very stable; <10% of the activity was lost upon 3 weeks of storage at 4°C.

DISCUSSION

Previous studies demonstrated that all secondary transport systems in *C. fervidus* use Na^+ instead of H^+ as coupling ions

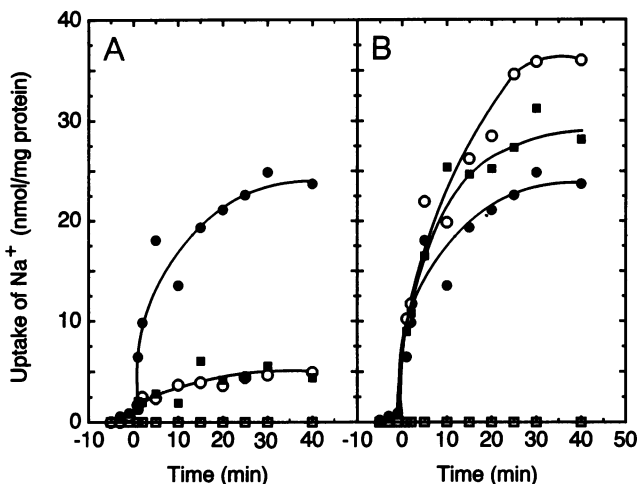


FIG. 5. Uptake of $^{22}\text{Na}^+$ into proteoliposomes. Uptake was performed at pH 7.0 and 45°C using 0.5 mM $^{22}\text{Na}^+$ (44.4 MBq/liter) and 3 mM Tris-ATP as substrates. (A) The following additions were made at time zero: \circ , No ATP; \bullet , ATP; \blacksquare , ATP plus 50 mM KNO_3 ; \square , ATP plus 3,5-di(*tert*)-butyl-4-hydroxybenzylidene malonitrile (SF-6847), monensin, valinomycin, and nigericin (1 μM each). (B) Effect of ionophores on $^{22}\text{Na}^+$ uptake. ATP was present in each assay. \bullet , No further additions; \blacksquare , 5 μM SF-6847; \circ , 5 μM valinomycin; \square , 1 μM monensin.

(2, 3). To use these secondary transport systems effectively, *C. fervidus* requires a ΔpNa^+ generating system. All attempts to detect a secondary Na^+/H^+ antiporter system failed. The observations that in intact cells only a $\Delta\psi$ and ΔpNa can be detected between pH 5.5 to 8.0 and that the ΔpH is close to zero also argue against the presence of a Na^+/H^+ antiporter. The results on the Na^+ efflux in intact cells and ATP-driven Na^+ uptake in proteoliposomes clearly demonstrate that an F/V-type ATPase generates the ΔpNa^+ . The concept of a Na^+ -ATPase as the sole Na^+ -translocating mechanism in *C. fervidus* is supported by the observation that substrates that can function as energy sources do not function as substrates of Na^+ -translocating decarboxylases but yield ATP by substrate level phosphorylation (G.S., unpublished).

Thermophilic organisms like *C. fervidus* are confronted with serious energetic problems since their cytoplasmic membrane is drastically more permeable for ions, including protons, at the optimal temperature of growth than the membrane of their mesophilic counterparts. *Bacillus stearothermophilus* compensates for this increased H^+ leakage by a very high respiratory chain H^+ -translocating activity (12). Also the thermophiles PS3 (13, 14), the anaerobic *Clostridium thermoaceticum*, *Clostridium thermoautotrophicum* (15, 16), and *Clostridium thermocellum* (17) use both Na^+ and H^+ as coupling ions. These organisms are able to grow over a wider pH range than *C. fervidus* but consequently are confronted with high maintenance energy requirements.

The use of a sodium instead of a proton motive force can be of important energetic advantage for *C. fervidus*. Phospholipid membranes are 6–10 orders of magnitude less permeable for Na^+ than for H^+ (18, 19). By using Na^+ as a sole coupling ion in vectorial energy-transducing processes and by avoiding H^+ cycling, metabolic energy can be spared. In doing so the organism has to pay a price since it is unable to regulate the internal pH, which narrows the pH range of growth. So far, the bacterium *C. fervidus* is unique by relying completely on Na^+ as coupling ion for primary and secondary transport processes. Although a primary Na^+ pump and a Na^+ -driven ATP synthase have been reported for *Propionigenium modestum* (20) and *Acetobacterium woodii* (21), the presence or absence of a Na^+/H^+ antiporter and the nature of the coupling ion in secondary solute transport processes are not known. In *P. modestum* the latter process is even supposed to be coupled to H^+ (5).

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